

# Role of retinoic acid and oxidative stress in embryonic stem cell death and neuronal differentiation

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**Abstract** Embryonic stem (ES) cells are a suitable system to study events occurring during development. In the present work we show that the apoptotic program was activated in ES cells, either by simple removal of the reducing agent 2-mercapthoethanol (2-ME), or by addition of *all trans*-retinoic acid (ATRA) to embryoid bodies. In these two conditions, there was an increase in reactive oxygen species and antioxidants such as catalase, superoxide dismutase or phenol prevented ATRA-induced cell death. Neuronal differentiation was observed when undifferentiated ES cells were treated with ATRA in the absence of serum and the presence of 2-ME.

**Key words:** Embryonic stem cell; Cell death; Neuronal differentiation; Retinoic acid; Oxidative stress

## 1. Introduction

Retinoic acid (RA) is a molecule with a broad biological activity present in all vertebrates. Clear effects of RA have been observed when systemically injected in pregnant mice [1], or when applied during specific processes such as limb and neural tube development [2,3]. Cellular responses to RA in vitro range from cell death to differentiation. RA-induced cell death with characteristics of apoptosis has been observed in several cell lines such as HeLa and HL-60 [4]. On the other hand, RA-induced differentiation has been observed, for instance, in embryonal carcinoma cells which differentiate into extra-embryonic endoderm-like cells or neuroectoderm derivatives such as neurons and astrocytes [5,6]. Although the ability of RA to regulate gene expression is well characterized, the mechanisms following this event are not known. In general, both RA-induced cell death and differentiation are mediated by specific nuclear receptors [7,8].

Oxidative stress has been proposed as a major mediator of apoptosis, since many agents which induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism [9]. Accordingly, in several systems, apoptosis can be inhibited by antioxidants and enzymes involved in the catabolism of reactive oxygen species (ROS), such as superoxide dismutase (SOD) and catalase [10–12]. In addition, Kane et al. [13] have shown that the expression of the antiapoptotic gene *bcl-2*, correlates with a decrease in the amount of ROS. Nevertheless, it has recently been shown that BCL-2 can also protect

from death in hypoxia [14,15], where ROS are not produced, consistent with the idea that oxidative stress acts as a transducing signal rather than being part of the death machinery.

In this work we found that mouse ES cells (AB1 line) are under constitutive oxidative stress, and that a high level in ROS correlated with apoptotic cell death. Embryoid bodies (EB) derived from ES cells underwent apoptosis in response to *all trans*-RA (ATRA), concomitant with a rise in ROS level. In agreement with the role of ROS in cell death, addition of catalase, SOD or phenol increased cell survival. ATRA also induced neuronal differentiation but, in contrast to other reports, EB formation was not required, and 2-mercapthoethanol (2-ME) favored the survival of differentiated cells.

## 2. Materials and methods

### 2.1. Cell culture and survival analysis

Undifferentiated ES cells were maintained on a fibroblastic feeder layer, as described by Robertson [5]. Culture medium was: DMEM complemented with 15% fetal bovine serum, 2 mM glutamine and 100  $\mu$ M 2-ME. To evaluate the role of 2-ME, undifferentiated cells were cultured at  $10^5$  cells/ml density, without the feeder layer, on 60 mm plates over-night. Plates were washed twice with PBS before removal of 2-ME and, 48 h later, cells were trypsinized (0.25% trypsin) 10 min, resuspended in PBS and trypan blue stained; blue versus white cells were scored. To form EB we followed the protocol previously described [5]. Briefly, ES cells at  $10^5$  cells/ml density were seeded on a bacterial plate in the presence or absence of 100 nM ATRA (Sigma) during 3 days. The formed cell aggregates were transferred to a graph tissue culture plate (where they attach) in medium without ATRA; a day after, the number of colonies formed, which is proportional to viability, were counted. Antioxidants were added simultaneously with ATRA to the following final concentrations: 100  $\mu$ g/ml (380 U/ml) bovine SOD (EC 1.15.1.1; Sigma); 1 mg/ml (25,000 U/ml) bovine catalase (EC 1.11.1.6; thymol free, Sigma); 0.0075% v/v phenol (Boehringer-Mannheim). SOD was denatured by incubation in boiling water during 30 min. Differentiation was evaluated in cells derived from EB, generated with or without ATRA, after they were cultured attached for several days as described by others [5,6,16].

### 2.2. DNA fragmentation and nuclei morphology analysis

In all cases DNA was extracted after 2 days of culture as follows. Cells were lysed with Lysis Buffer (10 mM EDTA, 50 mM Tris-HCl pH 8, 0.5% Sarcosyl, 0.5 mg/ml Proteinase K) during 1 h at 50°C. Then, 15 mg/ml RNase was added and incubated for 1 h at 50°C. Finally, fragmented DNA was enriched by centrifugation at  $13,000 \times g$  during 20 min. The supernatant was run on a 1.2% agarose gel, transferred to a nylon filter and revealed by hybridization with  $^{32}$ P-labeled total DNA. For nuclear morphology analysis, unfixed cells were stained with 20  $\mu$ g/ml DAPI (Molecular Probes) for 5 min and immediately photographed.

### 2.3. ROS quantification by DCF

Undifferentiated cells (with or without 2-ME, and with or without ATRA) or 7 days EB (generated with or without ATRA) were cultured on 24-wells plates for 48 h. Wells were washed twice with PBS before adding 1  $\mu$ g/ml 2,7-dichlorofluorescein diacetate (Molecular Probes), which is a compound that fluoresces when reacts with

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**Abbreviations:** ES, embryonic stem; 2-ME, 2-mercaptoethanol; ATRA, *all trans*-retinoic acid; EB, embryoid bodies; ROS, reactive oxygen species; SOD, superoxide dismutase; DCF, dichlorofluorescein diacetate; RA, retinoic acid.

ROS, such as superoxide, peroxide and hydroxyl radicals. Plates were read on a Cytofluor 2300 plate reader (Millipore) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm, after 15 min of incubation [13].

#### 2.4. Induction of neuronal differentiation

Undifferentiated cells were seeded directly on tissue culture plates with a defined medium (F12/DMEM, GIBCO; 25 µg/ml insulin; 100 µg/ml transferrin; 20 nM progesterone; 60 µM putrescin; 30 nM sodium selenite) and in the presence of 100 nM ATRA and 100 µM 2-ME. For the immunocytochemistry analysis, cells were fixed in methanol at  $-20^{\circ}\text{C}$  for 30 min and washed twice in PBS. The cells were incubated 1 h with a rabbit anti-150 kDa rat neurofilament antibody (Chemicon) diluted 1:400 in 0.1 M TBS (0.1 M Tris; 24.9 mM NaCl; pH 7.6), 0.5% non-fat milk and 1% Triton (TBS/M-T). The following rinses and incubations were performed: 3 rinses (10 min each) with TBS/M-T, 30 min incubation with biotinylated anti-rabbit antibody diluted 1:300 from Vectastain elite ABC kit (Vector Laboratories), 3 rinses (10 min each) with TBS/M-T, 30 min incubation with avidin-biotin complex, 3 rinses (10 min each) with TBS and developed with 3,3'-diaminobenzidine tetrahydrochloride (0.5 mg/ml) for 8 min.

### 3. Results

#### 3.1. ES cell death is associated with oxidative stress

2-ME is a common constituent of ES cell culture medium. Since 2-ME is a reducing agent, we reasoned that it is probably needed for survival by keeping the ROS levels low in ES cells. In agreement with this hypothesis, after three days of culture in the absence of 2-ME, 75% of the cells died, as shown in Fig. 1A. This death shared features of apoptosis such as nucleus fragmentation (not shown) and internucleosomal DNA degradation (Fig. 2A). The loss of viability correlated with a significant increase in ROS, as measured by DCF fluorescence (Fig. 3A). These results suggest a relationship between a burst of oxidative stress and the onset of ES cell death.

We also observed ES cell death when we added ATRA during EB formation, a condition in which ES cells undergo stochastic differentiation, even in the presence of 2-ME. The loss of viability in response to ATRA was estimated by the ability to form colonies in a tissue culture plate (Fig. 1B). As can be seen in Fig. 4, many cells looked apoptotic instead of differentiated. Accordingly, internucleosomal DNA degradation (Fig. 2A) and nucleus integrity, as determined by DAPI staining (Fig. 2B), were typical of cells undergoing apoptosis. To establish if ATRA-induced apoptosis is also mediated by oxidative stress, we quantified intracellular levels of ROS by DCF fluorescence. We found a significant accumulation of ROS in the presence of ATRA only when cells were grown as EB, the condition in which cell death was observed (Fig. 3B). To evaluate the contribution of ROS in the progression of cell death, we added enzymes involved in antioxidant pathways, such as SOD and catalase, to ATRA-treated cultures. At the third day, more colonies formed, than in cultures treated with ATRA alone, when SOD, catalase or SOD plus catalase were added (Fig. 1B). Addition of phenol to cultures treated with ATRA also increased the number of colonies formed (Fig. 1B).

In order to determine whether ATRA-induced death depends on events occurring during EB formation, ATRA was added to cells seeded directly in a tissue culture plate. In this condition, cells proliferated and formed colonies instead of dying (Fig. 5A); as expected, an increase in ROS was not observed (Fig. 2B). Therefore, ATRA is able to induce apoptosis only in cells that have formed or are forming EBs. The inability of undifferentiated ES cells to undergo ATRA-induced death was not due to the lack of response to ATRA; induction of neuronal differentiation by ATRA (described below), and the activation of ATRA responsive genes, such as the tissue non-specific alkaline phosphatase gene (Escalante-Alcalde observation), were observed.

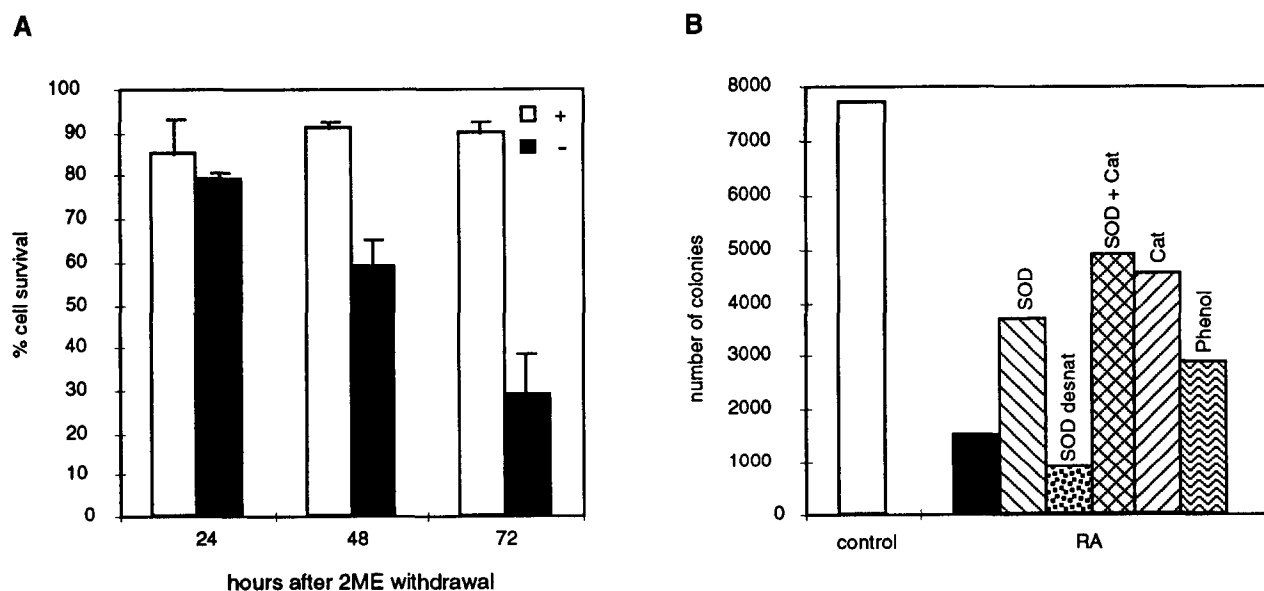


Fig. 1. Viability of ES cell in either the absence of 2-ME or the presence of ATRA. (A) Loss of viability by 2-ME withdrawal. Viability was determined by trypan blue exclusion; cells were grown with (+) or without 2-ME (-). The percentage of live cells with respect to the total number of cells is presented; a mean of 4 independent experiments is shown (error bars represent the standard deviation). (B) Loss of viability in the presence of ATRA during EB formation. Viability was determined at the third day of culture by the ability of EB cells to form colonies, which is proportional to the number of live cells. EB were grown in the absence (control) or presence of ATRA (RA), plus the indicated antioxidant: SOD, 100 µg/ml bovine SOD; SOD desnat, 100 µg/ml boiled bovine SOD; SOD+Cat, 100 µg/ml bovine SOD plus 1 mg/ml bovine catalase; Cat, 1 mg/ml bovine catalase; Phenol, 0.0075% v/v phenol. The average of two independent experiments is shown.

### 3.2. RA induces neuronal differentiation from undifferentiated ES cells

It has been reported that EC as well as ES cells can differentiate to neurons, when EB are treated with ATRA [5,16]. Using these conditions we were not able to see cells with neuron morphology. In addition, in cultures of undifferentiated ES cells (i.e. without the formation of EBs) treated with ATRA in the presence of serum, cells with differentiated morphology were not observed (Fig. 5A). Then, we decided to culture undifferentiated ES cells in a defined medium supplemented with ATRA and 2-ME for 2–5 days. In this latter condition, a mean of 15 neuron-like cells per mm<sup>2</sup>, immunopositive for neurofilament-M protein, were observed (Fig. 5B); these immunopositive cells were not observed in the absence of 2-ME (Fig. 5C). Therefore, serum inhibits differentiation in our system, and 2-ME may serve as a survival factor for the neurons obtained.

### 4. Discussion

In the present work we observed in ES cells a relationship between a burst in oxidative stress and the onset of apoptotic death, either by simple removal of 2-ME from the medium, or by addition of ATRA during EB formation. Gramzinski et al. (1990) have shown that certain embryonic carcinoma cell lines injected in the blastocoel die by oxidative stress, and that catalase is able to protect them [12]. This latter result is relevant, since ES cells are derived from the inner cell mass and, therefore, the line used in the present report may represent a cell population of the blastocyst sensitive to oxidative stress. However, the role of oxidative stress in ES cell death must result from a combination of factors, as we have not been able to reproducibly protect ES cells with other antioxidants (e.g. phe-

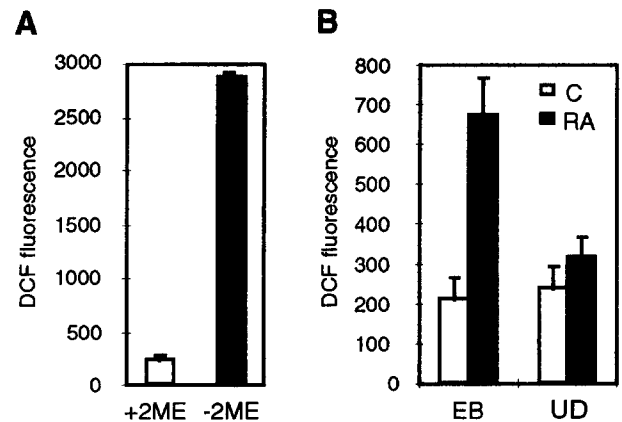


Fig. 3. Onset of cell death correlates with an increase in oxidative stress. (A) 2-ME prevents a burst of ROS in undifferentiated cells. (B) ATRA induces oxidative stress in EB cells (EB), but not in undifferentiated cells (UD). Cells were grown in the absence of ATRA (C) or in the presence of ATRA (RA); fluorescence units are arbitrary. The mean of 2 independent experiments done by quadruplicate are shown (error bars represent the standard deviation).

nol, tyrosine, *N*-acetyl-cysteine, glutathione, dithiothreitol). Intracellular iron concentration is one of such factors which 2-ME may affect reducing the iron regulatory protein [17].

#### 4.1. ATRA as activator of oxidative stress

Although several reports have shown the ability of retinoic acid (RA) to induce apoptosis, there is no precedent for the activation of oxidative stress in these systems. Zhang et al. [8] have shown that gene expression of tissue transglutaminase, an enzyme thought to keep the integrity of cell membranes during apoptosis, is activated by RA. However, the relevance of this data is questionable, as it is unlikely that this enzyme acts at the activation step of apoptosis. Recently, repression was suggested as the mechanism by which RA induces cell death [18]. If the latter hypothesis is true, it would be possible that genes coding for enzymes such as SOD or catalase are targets of RA repression. It is also possible that ATRA-induced death could be due to the induction of differentiation, such as the new phenotypes now require specific survival factors. Nevertheless, this latter hypothesis is unlikely because observed cell death occurred in the presence of serum (15% v/v).

EB formation induces initially the differentiation of two major cell types: the outer layer of endoderm, and the inner cells of ectoderm. During cavitation, ectoderm further differentiate to a pseudostratified epithelial layer. At the present time, we do not know which of these two cell types are affected by ATRA. Recently, Coucouvanis and Martin [19] showed that a signal which promotes ectodermic cell death is produced by the endoderm, and that the survival of the ectodermic inner layer depends on interactions with the extracellular matrix (i.e. the basal lamina). The cell population affected by ATRA in our experiments should be different from the one involved in cavitation, as morphology of ATRA-treated EBs were largely altered showing an abnormal basal lamina (data not shown).

Generation of oxidative stress through an ATRA signaling pathway may be a mechanism used during development to activate cell death. Digit formation in amniotes is an example where areas of cell death are well defined (called 'necrotic

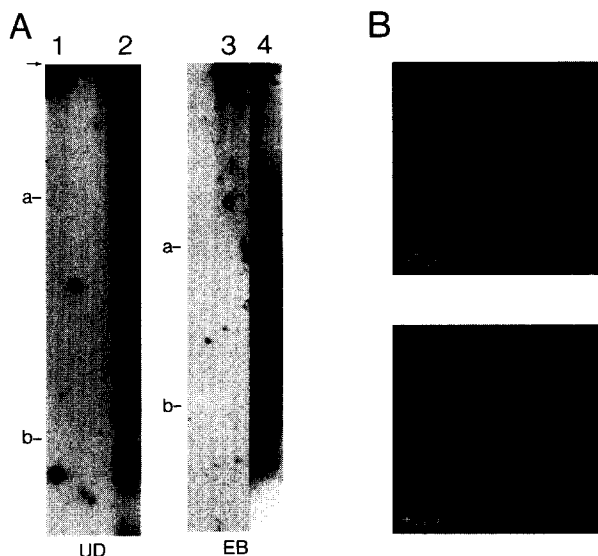


Fig. 2. ES cell death shares features of apoptosis. (A) Internucleosomal DNA degradation. UD, undifferentiated cells; 1, DNA from undifferentiated control cells; 2, DNA from undifferentiated cells grown in the absence of 2-ME; EB, embryoid bodies; 3, DNA from EB grown in the absence of ATRA; 4, DNA from EB grown in the presence of ATRA. Lambda DNA digested with *Hind*III was used as a molecular weight marker; only relevant sizes are shown (a, 2027 bp; b, 564 bp). The arrow indicates the origin of electrophoretic migration. (B) Nucleus morphology after staining with DAPI of EB cells treated with (+RA) or without ATRA (–RA).

zones'). ATRA increases the necrotic zones when applied to embryos [1] and, recently, it has been shown that ATRA induces cell death in explants of the interdigital region [20]. In agreement with our hypothesis, ATRA induces a subtle increase in ROS levels in primary cultures of limb cells (Salas-Vidal observation). Also, we have observed oxidative stress in the interdigits *in vivo*, as well as inhibition of interdigital cell death when limb explants were cultured in the presence of antioxidants (Salas-Vidal et al., submitted for publication).

#### 4.2. Neuronal differentiation by ATRA

In general, it has been proposed that to efficiently activate cell differentiation from ES cells it is necessary first to form EB. Then, EB can differentiate further within certain lineages by treatment with specific growth factors or substances such as DMSO or ATRA. Neuronal differentiation has been obtained using this protocol from ATRA treated EB of EC [6] or ES cells [16]. In contrast with these reports, we were able to induce neuronal differentiation from ES cells without the need of EB formation. Our success was due to the removal of serum and the addition of 2-ME in the differentiation medium. Serum was likely inhibiting differentiation by promoting proliferation, as it has been shown in other systems [21]. 2-

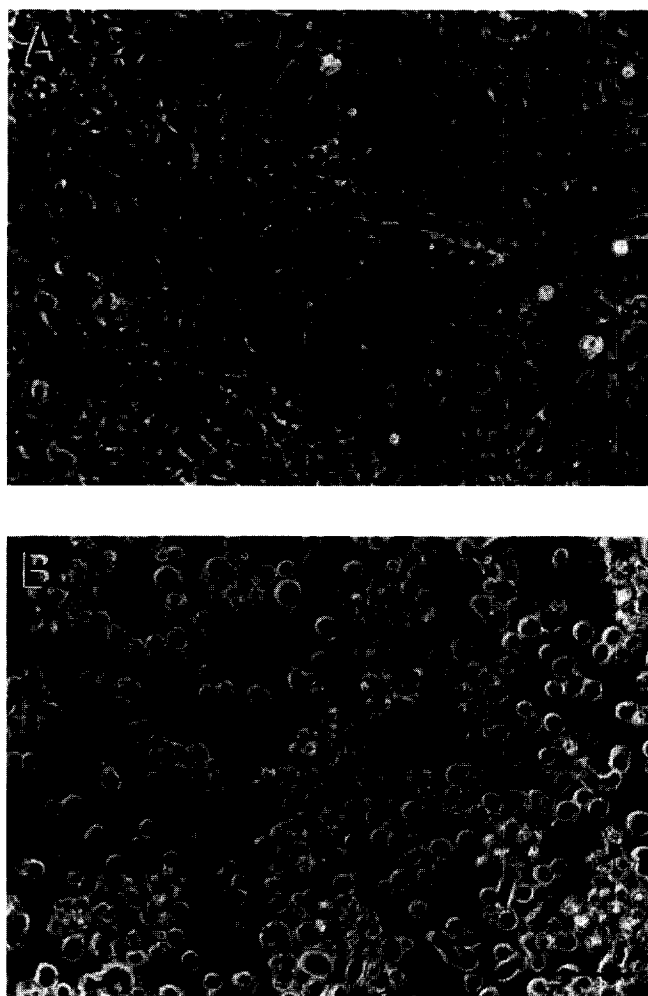


Fig. 4. Morphology of EB cells after seeding in a tissue culture plate. (A) EB formed in the absence of ATRA; (B) EB formed in the presence of ATRA. Refracting cells (a feature typical of apoptosis) appear only in the presence of ATRA. Bar = 50  $\mu$ m.



Fig. 5. ATRA-induced neuronal differentiation from undifferentiated ES cells. (A) Appearance of culture treated with ATRA in the presence of fetal bovine serum. In this condition cells survive and proliferate, but differentiated cells were not observed. (B) Neuron-like cells induced by ATRA in the absence of serum and the presence of 2-ME. Immuno-positive for neurofilament-M protein cells were analyzed by light bright microscopy. (C) Absence of immuno-positive cells for neurofilament-M protein in cultures treated with ATRA, without 2-ME in the medium. Bar = 50  $\mu$ m.

ME, on the other hand, could be substituting specific survival factors for the neurons obtained, perhaps through its reducing activity. The role of oxidative stress on death of neurons deprived of survival growth factors has been demonstrated in other systems [22,23]; specifically, 2-ME has been shown to support the survival and maturation of fetal mouse neurons [23]. The ability to induce neuron differentiation by simply adding ATRA directly to undifferentiated ES cells should be very useful, as it provides a short time-consuming experimental system for studies, such as the molecular analysis of neurogenesis, or the pre-screening of mutants affecting neuronal differentiation produced by gene-trap in ES cells.

In summary, we found that ATRA can elicit different responses in ES cells. ES cells die when ATRA is added during EB formation, but differentiate to neurons if added directly to undifferentiated cells. Apoptosis induced by ATRA may operate by a mechanism activated by ROS, as suggested not only by their presence, but also because ROS removal by SOD, catalase or phenol resulted in increased viability. Our system will be useful to determine the receptors involved and the mechanism by which ATRA alter ROS levels.

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